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Non-stimulatory peptides contribute to antigen induced CD8-TCR interaction at the immunological synapse

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Abstract

It is unclear if the interaction between CD8 and the TCR-CD3 complex is constitutive or antigen-induced. FRET microscopy between fluorescent chimeras of CD3 ζ and CD8 β showed that this interaction was induced by antigen recognition, within the immunological synapse. Non-stimulatory endogenous or exogenous peptides, presented simultaneously with antigenic peptides, increased the CD8-TCR interaction. This indicates that the interaction between the intracellular regions of a TCR-CD3 complex recognizing its cognate peptide-major histocompatibility complex (pMHC) antigen, and CD8 (plus Lck), is enhanced by a non-cognate CD8-MHC interaction. Thus the CD8 interaction with non-stimulatory pMHC helps mediate T cell recognition of antigen, improving the coreceptor function of CD8.

The $\alpha\beta$ T cell receptor (TCR) is responsible for the affinity and specificity of antigen recognition^{1,2}, whereas the coreceptors, CD8 or CD4, enhance the sensitivity of TCR recognition³. Disruption of coreceptor-pMHC interactions inhibit or change the quality of the T cell response^{3–5}. Coreceptors act in two main ways. Firstly, they bind to non-polymorphic regions of MHC⁴. This can aid in adhesion, but the main role is generally believed to be increasing the sensitivity of T cell activation via the entropic facilitation of TCR-pMHC binding, rather than through energetic stabilization of the tri-molecular complex^{6–9}. Secondly, CD4 and CD8 are associated with the kinase Lck. Coreceptor binding to pMHC recruits Lck close to the TCR, enabling it to phosphorylate components of the TCR's signaling complex (CD3), thus enhancing signal transduction³.

There are conflicting data on the interaction between the TCR-CD3 complex and coreceptors, and in particular whether any interaction between these molecules is constitutive or induced by antigen recognition. Various co-immunoprecipitation and flow cytometry fluorescence resonance energy transfer (FRET) experiments suggest a constitutive interaction between some TCR-CD3 and coreceptors in unstimulated T cells^{7,10–13}. Others show interaction only after T cell activation^{14–17}. Co-capping and co-modulation experiments also support an interaction of coreceptor with TCR^{18–21}. CD8 $\alpha\beta$ and CD4 reside on glycolipid-enriched microdomains or rafts, whereas TCR association with rafts is greatly increased upon stimulation^{22,23}. It is therefore questionable whether all of these assays measure a direct interaction or simply co-localization of the molecules on the same rafts.

Whether the interaction between CD8 and TCR is constitutive or antigen-induced has important consequences for the role of CD8. Constitutive association implies that CD8 acts as a universal amplifier in antigen recognition. Inducible interaction suggests an extra level of fine-tuning,

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Competing interests statement

The authors declare that they have no competing financial interests.

where CD8 could sharpen and amplify sensitivity and specificity of recognition. In order to address definitively whether CD8 and TCR interact, and to what extent the interaction is induced upon antigen recognition, we have used microscopy to measure FRET between CD8 β -yellow fluorescent protein (YFP) and CD3 ζ -cyan (C)FP during antigen recognition in live and fixed OT-I T-hybridoma cells. FRET can be used to address the question of proximity between molecules, because its effective range is less than 10nm.

T cells acquire a polarized morphology upon antigen recognition, in which certain surface and intracellular molecules become concentrated into the contact area between the T cell and antigen presenting cell (APC), known as the immunological synapse^{9,24}. This is a dynamic structure whose exact role in T cell activation is contentious, although it is without doubt the site of antigen recognition and signaling. The coreceptor is brought into the synapse quickly after T cell-APC contact^{25–27}. The CD8-MHC class I interaction is absolutely required for synapse formation, because a mutation in the CD8-binding site of MHC class I renders T cells unable to form T-APC conjugates²⁸.

FRET experiments performed by flow cytometry cannot relate molecular interactions between TCR and coreceptors to the formation of the immunological synapse. Microscopic evaluation of FRET between fluorescent chimeric proteins therefore has a great advantage in that it allows spatio-temporal localization of molecular interactions with subcellular resolution in living cells. This makes it possible to ask whether the molecular interaction between TCR and CD8 drives CD8 recruitment to the synapse, or if the interaction is induced in the synapse. Recent FRET microscopy experiments on living cells showed that interaction between fluorescent chimeras of CD4 and CD3 ζ was strongly induced by antigen recognition and occurred only in the synapse²⁶.

Certain APCs support non-antigen-specific recruitment of coreceptor to the synapse by pMHC bearing non-stimulatory or even antagonist peptide^{9,26,29}. Non-stimulatory, as well as antigenic pMHC class II, can contribute to T cell activation and synapse formation^{30,31}. This effect was noted at low antigen concentration, and was not caused by antagonist pMHC ligands³⁰. It involved interaction between TCR and the endogenous pMHC class II³¹.

Using FRET microscopy, we show that interaction between CD3 ζ and CD8 β is transiently induced at the synapse between a T cell and an APC loaded with agonist peptide, but not by a non-stimulatory peptide. The presence of non-stimulatory peptides enhances antigen recognition and increases CD8-CD3 ζ interaction. Thus the majority endogenous pMHC enhance recognition of the rare antigenic pMHC via a non-cognate interaction between CD8 and MHC class I.

RESULTS

Biological activity of fluorescent chimeric proteins

In order to study the interaction between TCR and coreceptors during antigen recognition in living cells, we developed fluorescent chimeras of CD3 ζ -CFP and CD8 β -YFP, expressing them in a T cell hybridoma. FRET between CFP and YFP reports when these molecules are brought within 10nm of each other^{26,32}. The OT-I hybridoma recognizes H-2K^b with an ovalbumin peptide (OVA)² and its TCR binds to K^b-OVA with relatively high affinity compared to non-stimulatory peptides for this TCR, such as a peptide derived from vesicular stomatitis virus nucleoprotein (VSV)³³. The C-termini of CD8 β and CD3 ζ were fused with YFP or CFP, respectively, with peptide linkers to allow proper folding and flexibility. The chimeric CD8 β -YFP and CD3 ζ -CFP genes were retrovirally transduced into OT-I hybridomas³⁴ expressing wild-type (WT) CD8 α , to obtain stable transfectants (OT-I.ZC.8 β Y) expressing surface CD8 β at amounts similar to CD8⁺ splenocytes (anti-CD8 β staining intensity: 738 $\pm$ 200 vs. 622

± 136 above background, respectively). CD8 β needs to pair with CD8 α for expression on the cell surface³⁵. CD8 β -YFP did not reach the surface of cells lacking CD8 α (data not shown), but was expressed on the surface of cells expressing CD8 α WT (Fig. 1a). Anti-CD8 α (or anti-CD8 β) staining showed complete co-localization and co-capping with CD8 β -YFP (Fig. 1b), confirming that CD8 β -YFP formed a complex with CD8 α . IL-2 release was undetectable in OT-I hybridomas lacking CD8. Transfection of CD8 α WT partially restored antigen reactivity, whereas CD8 β -YFP+CD8 α WT gave 1000-fold stronger responsiveness than CD8 α WT alone, similar to CD8 $\alpha\beta$ WT (Fig. 1c), showing that CD8 β -YFP retained the coreceptor properties of CD8 β . Immunoprecipitation with anti-CD8 β co-precipitated Lck (Fig. 1d), showing that the YFP moiety did not interfere with Lck binding to the cytoplasmic domain of CD8 α . This also confirms the CD8 α -CD8 β -YFP interaction, because Lck is associated with CD8 α . Therefore the CD8 β -YFP is biologically functional.

Antigen dependent and independent cell coupling

We quantitated the ability of antigenic or non-stimulatory peptides to induce stable conjugate formation, to assess the contribution of antigen recognition to the formation of stable conjugates^{26,29}. RMA-S cells were labeled with Cy5 and loaded with OVA or VSV peptides at concentrations that caused equal expression of stabilized K^b on the cell surface³⁶ (Fig. 2a). The OT-I.ZC.8 β Y cells were incubated with the RMA-S cells at 37°C. At different time points, the cells were pipetted to separate any weakly conjugated cells, and fixed. The percentage of OT-I.ZC.8 β Y cells forming conjugates with RMA-S cells was assessed by flow cytometry (Fig. 2b,c). In the absence of exogenously added peptides, less than 1% of the OT-I.ZC.8 β Y cells formed conjugates with RMA-S cells (Fig. 2b). However, when these few putative conjugates were sorted and visualized with a microscope, 90% were found to be single cells (data not shown). Therefore, RMA-S cells without exogenously added peptides rarely formed any stable conjugates with OT-I.ZC.8 β Y cells.

T cell-APC conjugate formation with RMA-S cells loaded with the non-stimulatory peptide VSV was increased compared to RMA-S without peptide (Fig. 2b). However conjugate formation was much stronger if the RMA-S expressed antigenic K^b-OVA (Fig. 2b). Thus, conjugate formation was strongly induced by an antigen recognition-dependent mechanism. The weak but significant activity of K^b-VSV suggested that non-stimulatory (e.g. endogenous) pMHC complexes could be important for initial formation of T cell-APC conjugates. The conjugates were sorted, imaged, and grouped according to whether they had recruited CD8 β -YFP with or without CD3 ζ -CFP to the T-APC interface. About 70% of T cells in conjugates recruited CD8 β -YFP, regardless of the peptide (Fig. 2d). This contrasted to CD3 ζ -CFP recruitment, which occurred mainly in cells forming conjugates with OVA-loaded APC, peaking around 15 min (Fig. 2d). Representative images are shown (**Supplementary Fig. 1** online).

CD8 recruitment is not peptide-specific

The relationship between CD8 recruitment and pMHC density was assessed by analyzing the synapses formed between OT-I.ZC.8 β Y cells and RMA-S cells expressing K^b-OVA or K^b-VSV. The intensity of CD8 β -YFP in the contact area was compared to CD8 β -YFP expression in the rest of the membrane (Fig. 3a). pMHC molecule expression on RMA-S cells was quantitated by comparing anti-H-2K^b-PE staining with reference beads loaded with known quantities of PE. When RMA-S cells expressing equally high amounts of K^b-OVA or K^b-VSV (26,000 molecules/cell) were added to the OT-I.ZC.8 β Y cells, a much smaller percentage of T cells formed conjugates with VSV (Fig. 2b). However, in T cell-APC conjugates, the same 2.5 fold increase of CD8 in the synapse relative to the rest of the cell membrane was observed with both OVA and VSV. Therefore conjugate formation depends on an early signal, that is infrequently generated in the absence of antigen. The equivalent ability of non-stimulatory and

agonist peptides to induce CD8 β -YFP clustering showed that CD8 recruitment itself was not purely an antigen-dependent process, but rather was driven by the CD8-MHC class I interaction. When K^b-OVA (Fig.3a) or K^b-VSV (**Supplementary Fig.2** online) were titrated, CD8 β -YFP recruitment decreased. Thus, the amount of CD8 recruitment depends on MHC class I density, whereas the cell-to-cell frequency of T-APC conjugate formation is antigen-specific.

In contrast, TCR-CD3 ζ recruitment to the synapse is antigen-dependent, as shown previously^{24,26,37} (Figs.2d,3b). In the presence of antigenic pMHC, CD3 ζ expression in the synapse was increased 1.5–1.7-fold compared to the rest of the cell membrane. This increased amount of CD3 ζ -CFP was not reduced as the level of K^b-OVA was reduced (below ~3000 K^b molecules per cell, too few T:APC conjugates were formed to analyze). The lower CD3 ζ -CFP level at the highest concentration of OVA was probably due to faster, stronger TCR downregulation at such high antigen density (see also Figs.4f,6). Thus, above a certain threshold, the degree of CD3 ζ recruitment was not dependent on MHC density *per se*.

Antigen induced CD8 β -CD3 ζ interaction

The interaction between two molecules (strictly, their molecular proximity) can be assessed by FRET imaging³². Upon CFP excitation, FRET leads to decreased fluorescence of CFP and increased fluorescence of YFP, as energy is transferred from CFP to YFP. OT-I.ZC.8 β Y cells were allowed to interact with OVA- or VSV-loaded RMA-S cells at 37°C, and the interaction of CD3 ζ -CFP and CD8 β -YFP was assessed by FRET efficiency imaging^{26,38}. Upon stimulation with OVA-loaded cells, both CD3 ζ -CFP and CD8 β -YFP were recruited to the synapse, where the FRET signal between CD8 β -YFP and CD3 ζ -CFP increased (Fig.4a,b). FRET intensity did not correlate with donor:acceptor ratio (Fig.4i), YFP intensity (Fig.4g,h), nor CFP intensity (Fig.4j). Thus, FRET between CD8 β -YFP and CD3 ζ -CFP was not due to non-specific, diffusion-driven interactions caused by increased crowding of the molecules.

The FRET signal was quantitated over time (Fig.4e). To get accurate time-points, and equivalent experimental conditions for FRET analysis, we fixed the conjugates, resulting in lower FRET efficiency compared to live cells. FRET peaked at 10–12 min after initiation of stimulation, showing that interaction between CD8 and TCR was transiently induced upon antigen recognition. The non-stimulatory peptide VSV did not induce an increase in the FRET signal (Fig.4c–e). It should be noted that basal CD3 ζ -CFP expression is high enough to give a FRET signal (Fig.4j), showing that the lack of extra CFP does not explain the failure to increase the FRET signal²⁶. A low basal FRET signal ($E_{app} \sim 2.5\%$) was detectable on the cell surface in the absence of any APC. Whether this was due to low constitutive interaction between CD8 β and CD3 ζ is unclear. TCR downregulation was assessed after stimulation of OT-I.ZC.8 β Y cells with RMA-S loaded with OVA or VSV. OVA induced rapid TCR downregulation whereas this was absent when VSV was used (Fig.4f). TCR downregulation and FRET signal downmodulation showed similar kinetics.

Immobilized pMHC induces CD8-CD3 ζ interaction

To study whether the interaction between CD8 β and CD3 ζ can be induced by pMHC complexes alone, we refolded soluble K^b with peptide *in vitro*, biotinylated the K^b molecule, and bound the complex to avidin-coated non-fluorescent beads. Both K^b-VSV and K^b-OVA-coated beads readily interacted with the T cells at 37°C, causing accumulation of CD8 β -YFP at the T cell-bead interface (Fig.5a,b). CD3 ζ -CFP became concentrated at the interface with K^b-OVA but not K^b-VSV-coated beads. Recognition of K^b-OVA caused rapid engulfment of the beads, as noted previously³⁹. The FRET signal was increased on the contact interfaces of K^b-OVA but not K^b-VSV beads (Fig.5a–c). Thus, CD8-CD3 ζ interaction was induced by pMHC complexes alone in the absence of costimulatory molecules.

Endogenous non-stimulatory peptides enhance antigen recognition

Endogenous pMHC complexes accumulate in the synapse between a CD4 T cell and APC, in the presence of agonist pMHC. This enhances T cell activation, indicating that simultaneous recognition of self and foreign peptide on the same APC can affect the response to a foreign ligand³⁰. To study how a non-stimulatory peptide influences antigen recognition and CD8-CD3 ζ interaction, we used the mAb 25-D1.16 that specifically recognizes K^b-OVA⁴⁰. This enabled comparison of T cell responses to RMA-S cells expressing various levels of K^b-OVA in the presence or absence of additional non-stimulatory ligand (K^b-VSV) (Fig.6a-c). The amount of OVA peptide was titrated and the amount of VSV peptide was varied so that the total MHC density on cells loaded with OVA+VSV was kept constant. Conjugate formation, TCR down-regulation and FRET efficiency were plotted as a function of anti-K^b-OVA fluorescence. Therefore, any difference in the dose response curves between the two groups was caused by the presence of the non-stimulatory pMHC. TCR down-regulation in response to OVA was enhanced by the presence of VSV (Fig.6a). Similarly, the formation of cell conjugates was greatly enhanced if the VSV peptide was also present (Fig.6b). Similar results were obtained at time points between 6–30 min (data not shown). Therefore, very small amounts of agonist peptide were needed to induce conjugate formation in the presence of excess non-stimulatory peptides. In the absence of non-stimulatory peptides, quite high amounts of the cognate antigen were required for conjugate formation. Most strikingly, the non-stimulatory peptide was able to increase interaction between CD8 and CD3 ζ , as measured by FRET (Fig. 6c). We performed similar experiments with six endogenous K^b-binding peptides that do not activate OT-I T cells⁴¹; P815, Mapk1p (Fig.6d-f), Stat3, Ndufa4, Slc2a3 and Hcph (Supplementary Fig.3d-f online). Similar to VSV (Figs.2,3), these peptides alone induced more T-APC conjugates compared to RMA-S cells without peptide, and were as active as OVA in recruiting CD8 to the synapse (Supplementary Fig.3b,c online). When used in combination with titrated amounts of OVA, these peptides, like VSV, enhanced conjugate formation, TCR downmodulation, and FRET. Thus, the ability of a non-stimulatory peptide to increase antigen recognition when presented simultaneously with OVA, was not limited to the viral-derived VSV peptide but also occurred with endogenous peptides. Thus, non-cognate binding of CD8 to MHC class I molecules bearing a non-stimulatory ligand, enhanced recognition of antigenic ligands.

DISCUSSION

There is considerable disagreement in the literature about whether the interaction between CD8 and the TCR-CD3 complex is constitutive or induced during antigen recognition. Interaction between TCR-CD3 and CD8 was detectable by co-immunoprecipitation from unstimulated cells, and therefore judged constitutive^{7,10–13}. Other data indicated a constitutive TCR-CD3-CD8 interaction that was increased by T cell activation^{15,16}. Immunoprecipitation analyses detect interactions between small proportions of the molecular species involved. These may not be representative of the majority of the molecules. Our FRET analysis showed that there was little interaction, as measured by FRET, between the CD3 ζ -CFP and CD8 β -YFP on unstimulated T cells. On antigen recognition by the TCR, interaction between CD3 ζ -CFP and CD8 β -YFP was triggered within the synapse, and an increased FRET signal was observed. Thus most of the TCR-CD3 and CD8 molecules do not interact in the absence of TCR stimulation, although we cannot say that there is absolutely no interaction. The low background FRET signal may represent such an interaction, but even so, the strong TCR-CD3-CD8 interaction induced in the immunological synapse by antigen recognition is striking.

Two recent experiments used FRET in flow cytometry to investigate TCR-CD8 interaction. In one case monomeric or tetrameric pMHC induced FRET between anti-CD3 ϵ and anti-CD8 bound to T cells¹⁷. In the absence of pMHC, no FRET was observed, indicating that the TCR-

CD3 interaction with CD8 was induced by the interaction of pMHC with both TCR and CD8. In the other case, FRET between anti-CD3 ϵ and anti-CD8 β $\pm$ saturating amounts of soluble monomeric pMHC was interpreted as showing constitutive TCR-CD3-CD8 interaction¹³. Potential reasons for the discrepancies between these and our results include the use of Abs (large, flexible molecules) as the fluorescent species in FRET experiments. These carry the inherent risk that the distance between the target molecules is much greater than the 10nm suggested by a FRET signal. Also, Abs could potentially induce cellular signaling. Previous experiments measured FRET between Abs to the extracellular portions of CD8 and TCR, whereas we measured FRET between intracellular portions of the molecules. The previous studies reflect on the ability of pMHC to couple TCR and CD8, whereas our results reflect more on the ability of cytoplasmic portions important in signal transduction to be brought together. Induction of FRET between directly fluorescent CD3 and CD8 by purified pMHC on beads confirms that pMHC is sufficient to induce the TCR-CD3-CD8 interaction¹⁷, and shows that this applies to the intracellular portions of the molecules.

The interaction between CD8 and CD3 ζ peaked within the first ten minutes and then gradually faded with time. The transient nature of the TCR-CD3-CD8 interaction may be important in regulating signal transduction. The similar kinetics of the FRET response, TCR downregulation and number of conjugates, suggests that the loss of the TCR-CD3-CD8 interaction is due to loss of TCR from the cell surface, and that as stimulation gets lower, the T cells dissociate from the APC.

We found non-antigen specific recruitment of CD8 into the synapse, as reported earlier for CD4²⁶. Antigen-independent synapses have been observed between naïve T cells and dendritic cells^{26,29}. We found that RMA-S cells presenting non-stimulatory peptide formed more T-APC conjugates than RMA-S cells without peptide (and therefore with little or no MHC class I). The frequency of conjugate formation was strongly increased in the presence of antigenic compared to non-stimulatory peptide. However, the non-stimulatory peptides induced CD8 clustering to the synapse as efficiently as the antigenic peptide in the conjugates. This indicated that CD8 clustering is driven by pMHC density and suggests that the weak CD8:MHC class I interaction⁶ is nonetheless sufficient to pull this ligand-receptor pair into the interface between two cells, as discussed previously⁴². This would have the effect of concentrating CD8 and pMHC molecules in the synapse where they can affect responses to antigen-stimulation. Clustering of CD8 to the synapse may explain earlier findings showing increased adhesion between CD8 and MHC class I molecules on CTLs, upon stimulation with soluble anti-TCR or antigen⁴³.

The TCR must recognize a few antigenic ligands in a vast excess of self-derived peptides. Such endogenous peptides mediate important survival signals and can enhance antigen recognition in CD4 T cells when presented simultaneously with an antigenic peptide³¹. So far, endogenous peptides have not been shown to aid antigen recognition by CD8⁺ T cells. Indeed, a prior attempt to demonstrate functional enhancement by endogenous peptides in CD8⁺ T cells failed⁴⁴. In this study, we found that the simultaneous presence of non-stimulatory (exogenous or endogenous) and agonist pMHC on the same APC enhanced CD8⁺ T cell responses. Non-stimulatory peptides presented simultaneously with an antigenic peptide increased interaction between CD8 and TCR-CD3, specifically between their cytoplasmic domains. Thus our FRET data demonstrate that the non-cognate CD8-MHC interaction enhances the interaction between CD8 and TCR during recognition of cognate pMHC.

The simplest model for coreceptor function has TCR and CD8 binding to the same antigenic pMHC, with the cytoplasmic region of coreceptor providing Lck to phosphorylate CD3. However, this classical model cannot explain how endogenous or other non-stimulatory peptides enhance antigen recognition. The classical model was initially questioned by

structural studies suggesting that it might be impossible for coreceptor to interact with the same MHC molecule as is contacted by the TCR⁴⁵ (although too little is known of the CD8 stalk to say this with any certainty). This suggested that coreceptor might bind pMHC with its extracellular domain, yet interact intracellularly with a TCR recognizing a different pMHC, thus bridging two TCRs. This “pseudodimer” model could explain how T cell activation requires TCR cross-linking to at least a dimer, yet an individual antigenic pMHC is sufficient to induce activation⁴⁶. The coreceptor must interact with the MHC molecule presenting antigen, suggesting that coreceptor interacts intracellularly with TCR binding to endogenous pMHC³¹. Experiments in MHC class II-restricted cells showed a role for TCR-recognition of the endogenous pMHC, because recruitment of endogenous pMHC was CD4-independent³⁰, and because only some endogenous pMHC worked to enhance T cell activation³¹. One model suggested that endogenous pMHC can amplify the T cell response to a few antigenic pMHC by virtue of Lck phosphorylation of TCRs binding transiently to the endogenous pMHC complexes⁴⁷. The few cognate TCR-pMHC interactions would recruit coreceptor plus Lck. Thus the Lck would be in the vicinity of the frequent (but very short-lived) TCR interactions with endogenous pMHC, and ready to phosphorylate these TCR complexes⁴⁷. An artificial heterodimer of agonist and certain endogenous pMHC complexes was sufficient to activate MHC class II-restricted T cells, suggesting that a TCR recognizing antigenic pMHC and one recognizing endogenous pMHC, are bridged by the coreceptor CD4³¹.

These models for amplification of recognition by endogenous pMHC could explain our data, except that we do not see a requirement for TCR interaction with endogenous pMHC. In our study on CD8⁺ cells, all seven non-stimulatory peptides enhanced antigen recognition. Only two of six peptides enhanced CD4⁺ antigen recognition strongly (plus two weakly)³¹. CD8 and CD4 are structurally very different⁴⁵, so the higher order structures formed by these molecules may be different. It is therefore possible that the MHC class I restricted response is not dependent on TCR interacting with endogenous pMHC in the same way as the MHC class II response. The non-cognate interaction between CD8 and MHC class I could enhance the cognate TCR-CD3-CD8 interaction (and therefore antigen recognition) by concentrating pMHC and CD8 to the synapse. Thus CD8-Lck is concentrated in the vicinity of any cognate interaction of TCR with antigenic-pMHC, making it easier for CD8 to bind antigenic-pMHC and thus to phosphorylate CD3 and start the T cell activation cascade. Also, pMHC is concentrated in the synapse so that the TCR has a more concentrated set of pMHC species to sort through to find the few antigenic-pMHC. These mechanisms would result in enhancement of coreceptor function, whether CD8 binds to the same pMHC as the TCR, or whether the CD8 associated Lck interacts in *trans* with ITAMs of CD3 ζ .

METHODS

Peptides and Antibodies

Peptides OVA (SIINFEKL), VSV (RGYVYQGL), P815 (HIYEFPL), Mapk1_{19–26} (VGPRYTNL), Stat3_{53–60} (ATLVFHNL), Ndufa4_{61–68} (VNVDYSKL), Slc2a3_{314–321} (VNTIFTVV) and Hcph_{503–510} (AQYKFIYV) were synthesized and purified as described^{33,41}. Anti-CD8 β (H35-17.2), anti-H-2K^b (AF6-88.5), anti-V β 5 (MR9-4), and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG were from BD Pharmingen (San Diego, CA).

Constructs and Cells

Hybridomas expressing the OT-I TCR $\pm$ CD8 α and β were made by retroviral transfection of TCR-deficient 58 $\alpha^- \beta^-$ cells³⁴. Chimeric genes for CD8 β -YFP (**Supplementary methods** online) and CD3 ζ -CFP²⁶ were constructed, inserted into retroviral vector pBMN-Z (S.

Kinoshita and G. Nolan, www.stanford.edu/group/nolan), and expressed in Phoenix packaging cells. The supernatant was used to transduce OT-I hybridomas (**Supplementary methods** online). For optimal FRET sensitivity and to avoid false negative results, the acceptor (YFP) should be in excess over donor (CFP). OT-I cell clones were selected based on optimal YFP/CFP molar ratio for FRET i.e. acceptor:donor molar ratio of 1:1 to 3:1.

Microscopy

Imaging was carried out with a dual camera system specifically designed for FRET imaging, allowing simultaneous acquisition of donor emission and acceptor emission during donor excitation, and fast changes between donor and acceptor excitation. This consisted of two CoolSnapHQ cameras (Roper, Tuscon, AZ), attached to a Zeiss 200M microscope through a beam-splitter (custom 510LPXR, Chroma) and stationary emission filters. Rapid wavelength switching was performed with a DG4 galvo illuminator customized with a 300W xenon lamp (Sutter, Novato, CA). YFP excitation was attenuated to 20% by appropriate positioning of the exit mirror. The system was run by Slidebook 4.0.3.9tz software (3I Corp., Denver, CO). The optical filters were: YFP excitation 510/20 (center/bandpass), YFP emission 550/50nm, CFP excitation 430/25, CFP emission 470/30, as well as Cy5 excitation 622/36, emission 700/75. Dichroic mirror was JP4. Exposure times were 0.2–0.5 s with 2x2 binning, 60x1.4 oil objective, and software flat field correction was used. 3D images were reconstructed from 15 z-sections 0.468 microns apart, collected with 60x1.4 oil objective with 1x1 binning. Live cells were imaged in HEPES–buffered 199 (low riboflavin autofluorescence) medium (Gibco, Grand Island, NY) with 5% FBS, no antibiotics, maintained at 37°C by the FCS2 live imaging chamber and objective heater (Bioptechs, Butler PA). T cells and APC were mixed and added into a prewarmed imaging chamber coated with poly-D-lysine (Sigma). For fixed cell imaging, cells were mounted in Slowfade Light antifade mounting media (Molecular Probes, Eugene, Oregon).

FRET analysis

A 3-filter set algorithm for the crosstalk compensation and extraction of donor-normalized FRET was used as described earlier^{26,38}. Details are provided (**Supplementary Methods** online). The FRET image was masked to accept only regions where CFP intensity was >4x noise level. Cells with CD8β-YFP/CD3ζ-CFP ratios outside the 1:1 to 3:1 stoichiometric range, as well as movement artifacts, were excluded from analysis. The average FRET was calculated from the synapse. Statistical differences were calculated using the mean difference hypothesis of Student's two tailed t-test assuming different variances and confidence level of 95%.

Antigen presenting cell preparation

RMA-S cells have a defect in the TAP2 gene, and cannot bind endogenous peptides to newly synthesized MHC class I molecules. Addition of synthetic peptides able to bind to K^b or D^b at low temperature leads to assembly and stable expression of pMHC on the cell surface. The peptide-loaded molecules remain stable at physiological temperature⁴⁸. Different peptides have different abilities to stabilize the cell surface pMHC. In order to have equal numbers of pMHC complexes on the cell surface for both OVA and VSV, we titrated the amount of peptide required to give equal pMHC loading by both peptides, as described³⁶. RMA-S cells were stained with Cy5 20h prior to experiments by incubating cells with 0.1 mg/ml of Cy5 monomeric succinimidyl ester (Amersham Biosciences, UK) in RPMI at RT for 5 min, washing with RPMI, and quenching with 10% FBS in RPMI. The RMA-S cells were incubated at 29°C overnight, pulsed with peptides for 30 min at 29°C, incubated at 37°C for 3h and washed once. For pMHC quantitation, RMA-S cells were stained with anti-H-2K^b-PE. QuantiBRITE PE fluorescence quantitation kit (Becton Dickinson) was used to calculate the number of molecules.

TCR down-regulation and conjugate formation assays

OT-I hybridomas and peptide pulsed RMA-S cells (1×10^5 each in 50 μ l) were incubated in flat bottom wells at 37°C. After incubation, the cells were stained for V β 5 and examined by flow cytometry. The data are shown as a percentage of V β 5 expression on the surface of cells compared to cells incubated with RMA-S without peptide, or as percentage of TCR endocytosed. For the conjugate formation assay, the cells were pipetted up and down three times after incubation, fixed in 2% paraformaldehyde, washed in PBS, and the paraformaldehyde inactivated by 10 mM Tris pH 7.4 in PBS. The cell conjugates were examined by flow cytometry based on simultaneous expression of YFP (OT-I hybridoma) and Cy5 (Cy5 labeled RMA-S cell).

Immunoprecipitation and Western blotting

Cells (10^7) were lysed in 1% Brij96, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM Na₃VO₄, and protease inhibitor cocktail, precleared with sepharose G beads and immunoprecipitated overnight with anti-CD8 β -conjugated beads. After washing, samples were run on 8% reducing SDS-PAGE gel, blotted and developed with anti-Lck (3A5; Upstate Biotechnology, Lake Placid, NY) followed by anti-MIgG-HRP.

Preparation of beads coated with pMHC

H-2K^b with a BirA biotinylation sequence and human β 2-microglobulin were individually produced in *E. coli*, refolded and biotinylated, as described^{33,36,49}. Biotinylated pMHC was added to streptavidin-coated 6 μ m carboxylated microspheres (Polysciences, Warrington, PA) at twice the saturating concentration, and incubated for 16h at 4°C with constant agitation. The beads were washed three times before use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Function of fluorescent chimeric proteins. **(a)** OT-I hybridomas with CD8α WT (broken line) or with CD8α WT + CD8β-YFP (solid line) were stained with anti-CD8β and analyzed by flow cytometry. **(b)** OT-I.ZC.8βY cells were stained with biotinylated anti-CD8α or -CD8β antibodies and crosslinked with streptavidin-allophycocyanin. **(c)** OT-I hybridomas lacking CD8 (▲), expressing CD8α WT (◆), CD8α WT + CD8β-YFP (●), or CD8α WT + CD8β WT (■) were cultured with irradiated B6 splenocytes loaded with titrated amounts of OVA. **(d)** OT-I hybridomas lacking CD8 (lanes 5,9), with CD8α WT (lanes 4, 8), with CD8α WT + CD8β WT (lanes 3, 7) or with CD8α WT + CD8β-YFP (lanes 2, 6) were immunoprecipitated with anti-CD8β (lanes 1-5, lane 1 is anti-CD8β Ab alone), and analyzed together with cell lysates (lanes 6-9) by SDS-PAGE, immunoblotted with anti-Lck and detected with anti-MIgG-HRP. These data are representative of ≥3 experiments, except (d), performed twice.

Figure 2.

T-APC conjugate formation and CD3 ζ and CD8 β recruitment to the synapse. **(a)** RMA-S cells were loaded with OVA (36 μ M, grey fill) or VSV (80 μ M, solid line) peptides, or no peptide (dotted line). These concentrations had previously been determined by titration to give equal H-2K^b density, as shown by anti-K^b Ab staining. **(b)** Time course of conjugate formation with antigen and non-stimulatory pMHC complexes. OT-I.ZC.8 β Y cells were allowed to interact with Cy5 labeled RMA-S cells expressing the same MHC density of either OVA or VSV peptide, or no peptide. At different time-points, cells were fixed and conjugate formation was assessed by flow cytometry. An example is shown in **(c)**. Results are representative of three independent experiments. **(d)** Proportion of conjugates that had recruited CD8 β -YFP or

CD3 ζ -CFP at different time-points. Conjugates were sorted by flow cytometry, visualized by microscopy, and randomized samples were categorized into three groups: increased CD8 β -YFP and CD3 ζ -CFP in synapse compared to the rest of the cell surface (gray bars), increased CD8 β -YFP only (filled bars), or no increase in either protein (open bars). The 0 min timepoint represents cells prior to conjugate formation. The results are averages of 34<n<108 cells from 2 independent experiments. See also **Supplementary Fig.1** online.

Figure 3.

CD8 clustering to the synapse is MHC-driven whereas CD3 ζ clustering is antigen driven. OT-I.ZC.8 β Y cells were allowed to interact with RMA-S cells expressing equivalent amounts of K^b-OVA or K^b-VSV complexes or different amounts of K^b-OVA. The number of pMHC molecules expressed by the RMA-S cells is indicated. OT-I.ZC.8 β Y cells were assessed for their fold increase in CD8 β -YFP in the synapse (**a**) or fold increase in CD3 ζ -CFP in the synapse (**b**), by comparing the fluorescence intensity in the contact area to that in the rest of the cell membrane. The results are shown as mean + SE, for n>10. In (**a**) p<0.05 between all the samples except for 26,000 OVA vs. 26,000 VSV and 7300 OVA vs. 3200 OVA. In (**b**) p<0.05 between VSV vs. all of the OVA samples, but not between the different OVA samples. Results are representative of three independent experiments.

Figure 4.

Agonist peptide induces co-localization and interaction between CD8β-YFP and CD3ζ-CFP. Live OT-I hybridomas expressing CD3ζ-CFP, CD8α WT plus CD8β-YFP (OT-I.ZC.8βY) were incubated with Cy5 stained APC loaded with OVA (a, b) or VSV (c, d). (a, c) mid-cell sections, (b, d) *en face* projections. Merged images (left) show CD8β-YFP (red), CD3ζ-CFP (green) and Cy5 (blue). FRET efficiency images are shown in the right column (see scale bar). Contact areas were defined in 3D by the T cell-APC fluorescence overlap. (e) OT-I.ZC.8βY cells were incubated with OVA or VSV loaded RMA-S cells for indicated times, fixed and average FRET efficiency + SE (18 < n < 38, except OVA 30 min n = 13, VSV 20 min n = 11, VSV 30 min n = 15) was assessed from the synapse. *** $p < 0.00002$; ** $p < 0.01$ between OVA and

VSV. For VSV there were too few 5 min T-APC conjugates to measure. **(f)** As **(e)**: cell surface TCR was assessed with anti-V β 5. **(g-j)** OT-I.ZC.8 β Y cells were incubated with OVA **(g,i,j)** or VSV **(h)** loaded RMA-S cells for 12 min and FRET efficiency was assessed from the synapses. **(g,h)** FRET efficiency versus YFP intensity, **(i)** FRET efficiency versus YFP/CFP ratio (The molar ratio= intensity ratio/2.2), **(j)** FRET efficiency versus CFP intensity.

Figure 5.

Close molecular proximity of CD8β and CD3ζ can be induced by pMHC molecules alone. OT-I.ZC.8βY cells were incubated with K^b-OVA coated beads (**a**) or K^b-VSV coated beads (**b**). The beads are not themselves fluorescent. K^b-OVA induced the recruitment of both CD8β-YFP and, to a lesser extent, CD3ζ-CFP (**a**), whereas K^b-VSV allowed only the recruitment of CD8β-YFP to the contact interface with the bead (**b**). The cells almost engulf the beads, as seen previously³⁹. The FRET signal was specifically induced at the interface with K^b-OVA beads but not with K^b-VSV beads. (**c**) statistics of fixed cells are shown. The results are shown as average FRET efficiency + SE ($n=36$ for OVA coated beads, $n=36$ for VSV coated beads, $n=19$ for OVA RMA-S and $n=10$ for VSV RMA-S). $p=2.7 \times 10^{-6}$ between OVA and VSV beads, $p=0.032$ between OVA beads and VSV RMA-S, and $p=0.123$ between OVA beads and OVA RMA-S.

Figure 6.

Non-stimulatory peptides increase antigen induced interaction between CD8 and TCR. RMA-S cells were loaded with different amounts of OVA (◆), OVA+VSV (○), OVA+P815 (□), or OVA+Mapk1 (Δ). The amount of K^b-OVA was determined using anti-K^b-OVA Ab 25-D1.16⁴⁰ (x-axis). The total number of K^b molecules for the OVA-only group was estimated from staining with PE labeled anti-K^b (AF6-88.5), as in Fig.3. 26,000 K^b molecules corresponds to 3515 MFI on the K^b-OVA scale, 7300 K^b to 987 MFI and 3200 K^b to 433 MFI. OVA+VSV, +P815 or +Mapk1-loaded RMA-S cells all expressed ~26,000 K^b molecules. (a,d) OT-I.ZC.8βY cells were incubated with OVA or OVA+VSV, +P815 or +Mapk1 loaded RMA-S cells for 1h and stained with anti-Vβ5. (b,e) OT-I.ZC.8βY cells were incubated with Cy5 labeled RMA-S cells for 12 min, fixed and conjugate formation assessed by flow cytometry. (c,f) FRET efficiency was assessed from synapses. Results are shown as average FRET efficiency +SE (17<n<96 (c), 12<n<48 (f), n_{average}=30). ★ FRET efficiency values significantly different between OVA and OVA+VSV groups (c), or between OVA and OVA+P815 groups (f). * significantly different between OVA and OVA+Mapk1 groups (f). (For clarity, not all significant differences are marked).